Q: What is the best solvent for pimonidazole HCl (Hypoxyprobe-1)?

A: Pimonidazole HCl is the hydrochloride salt of the weak base, pimonidazole, and is very soluble in aqueous solutions including neutral buffered saline (116 mg/mL or 400 millimolar). This permits the use of small volumes (0.1-0.5 mL) for intraperitoneal or intravenous injections of pimonidazole HCl in animal studies.

Q: What dose of pimonidazole HCl) should be used for hypoxia marking?

A: The extent of pimonidazole binding in hypoxic tissue will depend on the rate of bioreductive activation and on tissue exposure to pimonidazole (exposure = concentration x time). It is hard to predict the rate of reductive metabolism but the effect of exposure can be examined. In the following calculations, concentrations are given in terms of pimonidazole hydrochloride (MW 290.7).

Strong hypoxia staining is observed in spheroids exposed in vitro to 58mg/kg (concentration in medium) for 1 hour. Exposure = 58mg/kg x 1 hour = 58mg/kg-hour.

Strong staining for hypoxia in tumor tissue and in normal epithelia is obtained in humans with a dose of 0.5gm/m2 (ca 14mg/kg) where the plasma half-life of pimonidazole is 5 hours. Exposure = 14 mg/kg x 5 hours = 70 mg/kg-hour.

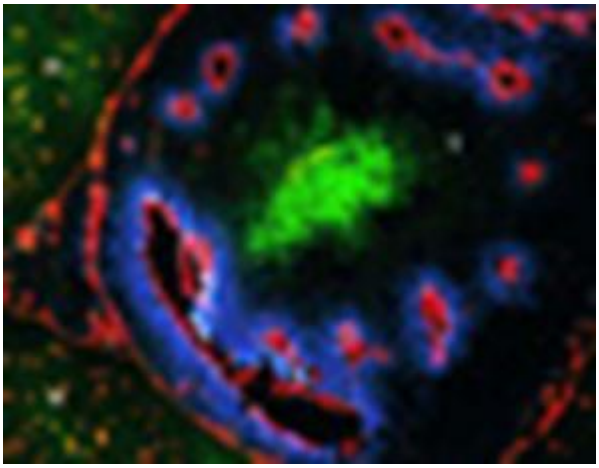
Strong staining for hypoxia in mouse tumor tissue is obtained with 60 mg/kg where the plasma half-life of pimonidazole is 0.25 hours. Exposure = 60mg/kg x 0.25 hours = 15 mg/kg - hour.

In summary, for small animals of uniform size such as laboratory rats and mice, a dose of pimonidazole HCl of 60 mg/kg body weight is recommended as a good balance between effectiveness and economy. Doses ranging from 30 mg/kg to 400 mg/kg have been used in mice and rats without toxicity or altered oxygen levels due to blood flow effects with the exception that blood flow effects have been observed at doses above 100 mg/kg of pimonidazole for tumors implanted in the hind legs of mice. Caution must be taken, therefore, when doses > 100 mg/kg are used in hind leg tumor models.

For larger animals with non-uniform body size, the dose is typically calculated on the basis of surface area. For humans, the recommended dose is 0.5 gm/m2 while for dogs a dose of 0.28 gm/m2 has been used.

Q. Does Hypoxyprobe™-1 penetrate hypoxic brain and brain tumor tissue?

A: Although Hypoxyprobe™-1 is water soluble, its corresponding free base has an octanol water coefficient of 8.5 and, as a result, the marker freely penetrates into both brain and brain tumor tissue.



Immunofluorescence staining of a frozen section of a rat brain tumor. Vasculature: ME 9F1; Red. Hypoxia: Pimonidazole adducts; Green. Perfusion: Hoechst 33342; Blue. Normal brain (N) and tumor tissue (T). Original magnification was x 200. Note the regular pattern of vasculature in normal brain versus poorly organized vasculature in tumor tissue (Bernsen et al, Journal of Neurosurgery 93: 449-454, 2000; by permission).

Q. Is pimonidazole HCl the best probe for detecting hypoxia in vivo?

A: Pimonidazole HCl, the old standard immmunohistochemical hypoxia marker, has real advantages including high water solubility (116 mg/mL in saline) that allows administration as small volume injections ip or iv. Markers such as the hexafluorinated CCI-103F have aqueous solubility of 10 millimolar or less and are usually administered as ip emulsions of peanut oil and DMSO in order to avoid hemodilution.

Solid pimonidazole HCl is very stable (years) when stored at room temperature or at 4oC. Concentrated aqueous solutions of pimonidazole HCl are very stable (years) when stored at 4oC in the absence of light. Mouse and rabbit antibodies to pimonidazole adducts are stable for ≥ one year stored at 4oC.

LD50(7days) for pimonidazole in mice is 728 mg/kg classifying it as a non-hazardous chemical of low toxicity.

Although pimonidazole HCl is very water soluble, pimonidazole as a free base has a high octanol-water partition coefficient of 8.5 and it readily penetrates all tissues including brain. In fact, it accumulates in most tissues 3 fold above plasma levels so that its effective tissue concentration is much higher than other 2-nitromidazole hypoxia markers.

Pimonidazole binding can be detected by a wide range of techniques that include: immunofluorescence in frozen fixed tissue sections; immunoperoxidase staining in formalin fixed paraffin embedded tissue sections; ELISA; or, flow cytometry.

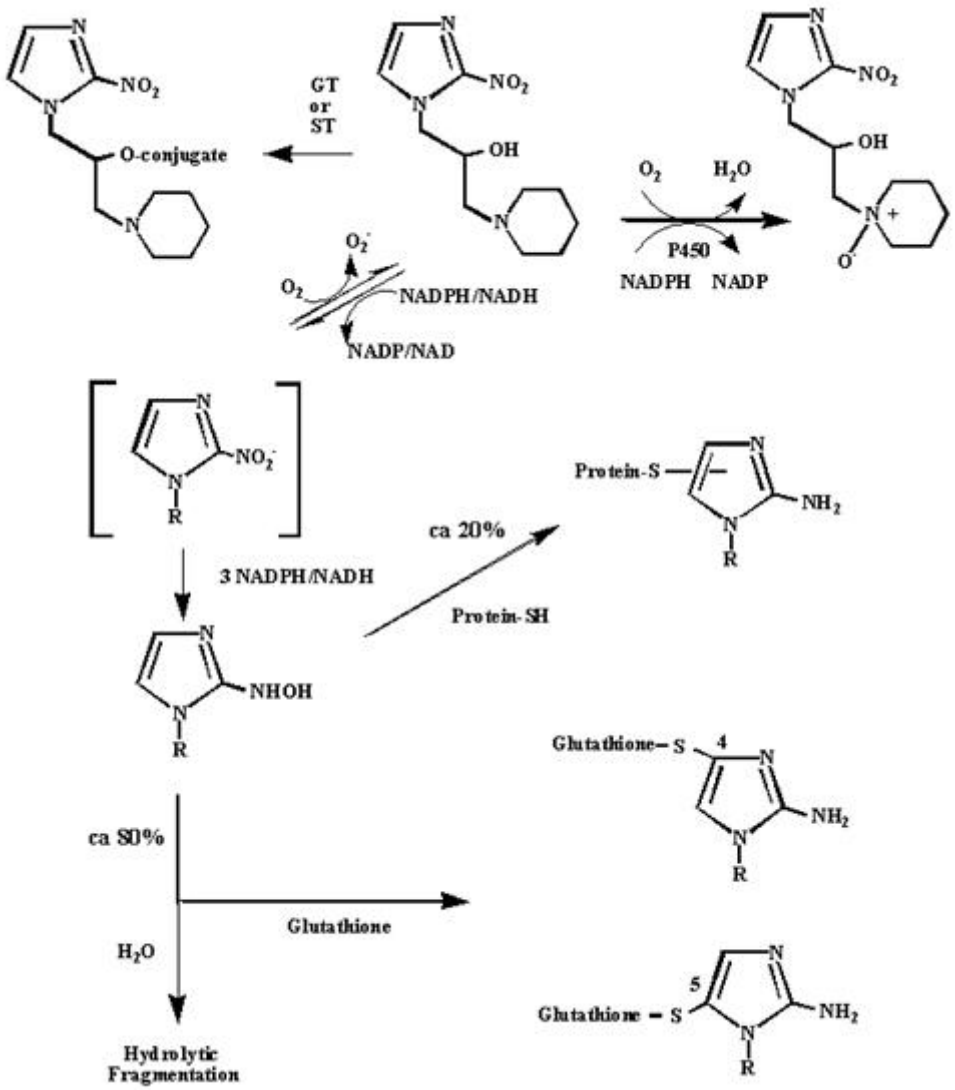
Q. Can the monoclonal antibody to Hypoxyprobe™-1 adducts be used on mouse tissue?

A: Yes. For formalin fixed paraffin embedded tissues we recommend a peroxidase F(ab)2

secondary antibody strategy. This gives a very clean background and is applicable to a variety of animal species.

Q. What is the mechanism for the activation and binding of pimonidazole to hypoxic cells?

A: Varghese et al. showed that hypoxic cells bind 2-nitroimidazoles to peptide thiols such as glutathione. The current view of the metabolism of pimonidazole is summarized in the scheme below. O2 competes for the addition of the first electron to pimonidazole which accounts for the pO2 dependence of activation and binding. On the basis of test tube experiments, it is estimated that ca 20 % of activated pimonidazole binds to cellular thiols and ca 80% does not bind but is fragmented by reaction with water. Pimonidazole is subject to oxidative metabolism leading to easily excretable N-oxide, sulfate and glucuronate derivatives (ST = sulfotransferase; GT = glucuronly transferase). These oxidative pathways do not appear to interfere with the utility of pimonidazole as a hypoxia marker.



Q. What is the concentration of the IgG1 monoclonal antibody in the antibody solution supplied with the Hypoxyprobe kits?

A: The concentration of the mouse IgG1 monoclonal antibody in the exhausted hybridoma

solution supplied in the Hypoxyprobe-1 Kit is ca 60 micrograms/mL

The concentration of affinity purified IgG1 in the FITC-conjugated MAb in the Hypoxyprobe-1 Plus Kit; the Hypoxprobe-1 Green Kit; the Hypoxyprobe-1 Red549 Kit; the Hypoxyprobe-1 RedAPC Kit; and the Hypoxyprobe-1 Biotin Kit is ca 500 micrograms/mL.

The concentration of active IgG molecules in affinity purified rabbit antisera to pimonidazole adducts and the unpurified rabbit antisera to Hypoxyprobe-F6 (CCI-103F) adducts is not known.

Q: Does pimonidazole binding detect chronic and acute hypoxia?
A: Two categories of hypoxia occur in solid tissues – diffusion-limited chronic hypoxia and perfusion-limited acute hypoxia. Chronic hypoxia arises at the distal end of oxygen gradients created by oxygen consumption in cells close to blood vessels compounded, in the case of tumors, by deficiencies in local oxygen supply arising from longitudinal gradients of pO2 in vascular trees (see Dewhirst et al., Temporal changes in pO2 of R3230AC tumors in Fischer-344 rats, Int J Radiat Oncol Biol Phys 42: 723-726, 1998). The presence of chronic hypoxia implies that cells in tissues consume oxygen at a rate that is independent of oxygen supply thereby driving pO2 to very low levels in microregions distal to blood vessels. That is, most cells possess characteristics of a 揜egulating cellular phenotype that is preprogrammed to adapt to low pO2 by smoothly transitioning to glycolytic based energy production (see Hochachka, Patterns of O2-dependence of metabolism, Adv Exp Med Biol 222: 143-151, 1988).

In contrast to chronic hypoxia with static, metabolically controlled pO2 gradients, acute hypoxia is associated with fluctuating pO2 that results from blood flow instabilities which, in the case of tumors, is created by transient vascular occlusion. Acutely hypoxic tumor cells, being proliferative, might be more therapeutically relevant than quiescent, chronically hypoxic cells. In normal tissues, fluctuating hypoxia is associated with hypoxia-reperfusion injury. With respect to whether pimonidazole can detect both chronic and acute hypoxia, compounds that incorporate weakly basic substituents (pKa $\geq$ 8.0) are concentrated in tissues ca 3 fold above circulating blood levels. This property of weakly basic compounds is based on the effect of differentials in intra- and extracellular pH on intracellular concentrations of weakly basic compounds. In particular, at pH 7.4 weakly basic 2-nitroimidazoles are concentrated intracellularly 2-fold compared to extracellular concentration. This concentration increase is directly reflected in increased hypoxic cell radiosensitization and labeling with hypoxia markers. Because cells experiencing fluctuating hypoxia are proximal to blood vessels and at relatively high pH, weakly basic, 2-nitroimidazole hypoxic markers such as pimonidazole are concentrated in these cells whereby episodes of acute hypoxia lead to higher levels of binding compared to hypoxia markers lacking weakly basic moieties. In this way, pimonidazole and its analogues are superior for detecting acute hypoxia (see Kleiter, et al. A comparison of oral and intravenous pimonidazole in canine tumors using intravenous CCI-103F as a control hypoxia marker. Int J Radiat Oncol Biol Phys, 64: 592-602, 2006 for further discussion and literature cited).

Q. How soon after pimonidazole HCl administration can tissues of interest be harvested?
A: Tissues become anoxic during harvesting and binding of circulating pimonidazole during

tissue harvest might, in principle, give false measures of hypoxia. The answer lies in comparing tissue exposure to pimonidazole during labeling and harvesting periods. Exposure (pimonidazole concentration x time at 37oC) is great during the labeling period and extremely short during the harvesting period because pimonidazole concentration is limited to that in the tissue at the time of harvest. A combination of low marker concentration, rapid harvest and immediate fixation in cold medium will eliminate measurable levels of non-specific binding.

In human tumor studies, biopsies are generally taken 16-24 hours after pimonidazole HCl infusion. The plasma half-life of pimonidazole in humans is ca 5 hours so that 16 to 24 hours represents 3 to 5 plasma half-lives of circulating pimonidazole. This means that 1/8 to 1/32 of the initial concentration of pimonidazole is present at the time of harvesting. This, combined with rapid transfer of biopsy material to cold fixative, minimizes non-specific pimonidazole binding as shown by low background binding in the majority of cells close to blood vessels.

In some human tumor studies, biopsies were taken 1.5 to 4 hours after pimonidazole HCl infusion and biopsies immediately fixed in liquid nitrogen. This approach also gave low background binding in cells near blood vessels. Although the level of circulating pimonidazole is relatively high 1.5 to 4 hours after infusion, the exposure to pimonidazole in harvested tissue was extremely short compared to exposure to pimonidazole during the in vivo labeling period.

The experience with human tumors can guide experimental studies. The plasma half-life of pimonidazole in mice is typically 0.25 hours. Under these circumstances, a harvest time of 1-2 hours combined with rapid addition to cold fixative effectively eliminates non-specific binding. This conclusion is particularly important for experiments involving carbon dioxide asphyxiation where the duration of global hypoxia is poorly defined. More rapid euthanasia techniques lend themselves to shorter times of harvest as long as rapid tissue harvest and fixation in cold fixative is carried out.

Q. On what basis is the pO2 threshold ≤ 10 mmHg set for pimonidazole binding?

A: It is difficult to measure Km(O2) for 2-nitroimidazole binding in solid tissue. The best experiment to date is Gross et al.担 comparison between oxygen microelectrode measurements of pO2 and misonidazole binding as measured by autoradiography of radioactively labeled misonidazole in the spheroid model of solid tissue. Grain densities due to misonidazole binding increased steeply below 10 mm Hg (Gross et al. Calibration of misonidazole labeling by simultaneous measurement of oxygen tension and labeling density in multicellular spheroids, Int. J. Cancer 61: 567-573, 1995). Chou et al. found that the Km(O2) for pimonidazole binding is similar to that for misonidazole in HeLa cells and concluded that 10 mm Hg is also a reasonable threshold value for pimonidazole binding in solid tissue (Chou et al. Evidence that involucrin, a marker for differentiation, is oxygen regulated in human squamous cell carcinomas. Br. J. Cancer, 90: 728-735, 2004).

A feature of solid tissues that is absent in sparse cell cultures is that oxygen consumption creates very steep O2 gradients so that the distance over which different Km(O2)s are traversed is foreshortened. For example, steep pO2 gradients are observed in liver tissue wherein

immunostaining for pimonidazole adducts goes from background to intense staining over a few cell diameters. Consistent with the presence of steep pO2 gradients in tissues are the immunostaining patterns for oxygen regulated proteins such as involucrin and carbonic anhydrase IX that closely resemble those for pimonidazole binding even though the Km(O2) for oxygen regulated proteins is ca 15 mm Hg compared to 2-4 mm Hg for pimonidazole binding in vitro (see Chou et al. Evidence that involucrin, a marker for differentiation, is oxygen regulated in human squamous cell carcinomas. Br. J. Cancer, 90: 728-735, 2004 for further discussion).

Q. Does in vivo N-oxidation affect pimonidazole as a hypoxia marker?

A. The piperidine moiety in pimonidazole is easily oxidized to its N-oxide metabolite. This, in principle, could impact the effectiveness of pimonidazole as a hypoxia marker. (See Arteel et al. Reductive metabolism of the hypoxia marker pimonidazole is regulated by oxygen tension independent of the pyridine nucleotide redox state. Eur J Biochem, 253: 743-750, 1998 for a detailed discussion of the metabolism of pimonidazole). In vivo, pimonidazole N-oxide is formed via the action of flavin mono-oxygenases (FMO). FMO isoform distribution varies among species producing different plasma levels of N-oxide. Interestingly, the route of pimonidazole administration (iv or oral) has little impact on plasma levels of N-oxide (see Kleiter et al. A comparison of oral and intravenous pimonidazole in canine tumors using intravenous CCI-103F as a control hypoxia marker. Int J Radiat Oncol Biol Phys, 64: 592-602, 2006 for further discussion). Strong oxidants such as peroxynitrate formed by the reaction of superoxide anion with nitrous oxide (NO) can also oxidize pimonidazole. However, the formation of N-oxide does not appear to compromise the effectiveness of pimonidazole as a hypoxia marker.

First, N-oxide formation is reversible by the reducing action of heme-iron complexes in blood and by tissue reductases such as xanthine dehydrogenase and reduced cytochrome P-450 whereby the loss of pimonidazole by oxidation is limited (see Walton et al. The reversible N-oxidation of the nitroimidazole radiosensitizer Ro 03- 8799. Biochem Pharmacol, 34: 3939-3940, 1985).

Second, it has been shown by the use of a second hypoxia marker, that the extent of hypoxia marking by pimonidazole is independent of pimonidazole plasma concentrations at the concentration recommended for hypoxia marking.

Third, because there is no cross reactivity between pimonidazole and its N-oxide derivative for anti-pimonidazole antibodies, the N-oxide does not interfere with the detection of pimonidazole adducts in hypoxic tissues (see Kleiter et al. A comparison of oral and intravenous pimonidazole in canine tumors using intravenous CCI-103F as a control hypoxia marker. Int J Radiat Oncol Biol Phys, 64: 592-602, 2006 for further discussion).